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(54) Title: PRODUCTION OF PROTEINS USING BACILLUS INCAPABLE OF SPORULATION

(57) Abstract

The invention provides a method of obtaining bacteria of the genus *Bacillus* other than *B. subtilis* incapable of sporulation by using information from *B. subtilis* to mutate a parent strain to be truly asporogenous. Such strains are used for the production of useful metabolites, specifically polypeptides, such as enzymes.

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PRODUCTION OF PROTEINS USING BACILLUS INCAPABLE OF SPORULATION

FIELD OF THE INVENTION

The present invention relates to a process for the production of various products, especially translocated polypeptides by using bacteria of the genus *Bacillus* which have been mutated to be incapable of sporulation, methods for producing such bacteria, and DNA constructs to be used in the processes.

BACKGROUND OF THE INVENTION

Bacteria of the genus *Bacillus* are being used in the production of various polypeptides and proteins for use in medicine and various industries.

As an example the species *Bacillus licheniformis* is used extensively for the production of industrial enzymes such as amylase and protease and is a popular host for the industrial preparation of cloned gene products (1, 2, 3). Hundreds of tons of extracellular enzymes are manufactured annually from this organism resulting in the need to dispose of a considerable tonnage of spent organisms, usually as composts.

From the environmental viewpoint, it is important that this material is dead when distributed to soils. This is generally achieved by treatment of the sludge with chemicals, radiation and/or heat.

Although most production strains are Sp^-, the lesions giving rise to this phenotype have usually been introduced during random mutagenesis exercises and are of unknown function and efficiency. The possibility that low levels of spores are present in the waste biomass is therefore high and complicates the sterilization process by necessitating harsher, more expensive, and environmentally less acceptable killing conditions to ensure spore eradication.

Moreover, the introduction of stricter legislation governing production strains, makes the use of a well-defined sporulation mutant highly desirable. Such a mutant should preferably be;

(i) totally defective in sporulation,

(ii) completely stable and unable to revert to sporogeny, and

(iii) similar or superior to the parent in the synthesis and secretion of extracellular enzymes.

The genetics of sporulation in *B. subtilis* is now at an advanced stage (4, 5) and, from the sporulation genes of *B. licheniformis*, which have been well characterized (17, 6, 7) it seems likely that this close relative of *B. subtilis* will follow a similar, if not identical, developmental pathway.

The first morphological change observed during the sporulation process is the synthesis of an asymmetric septum at stage II. The smaller daughter, which will ultimately become engulfed by the mother cell, gives rise to the prespore. Gene expression in the mother cell and maturing spore is partly governed by the ordered synthesis and activation of a cascade of sigma factors, which direct RNA polymerase to transcribe sporulation specific promoters in a temporally and spatially oriented fashion (4, 5).

The σ^F protein, the product of the *spoIIAC* gene (8, 9) is one such sigma factor which is present in the predivisional cell but its activity is restricted to the prespore and only becomes evident after septation (14). This sigma factor is crucial for establishment of compartment-specific gene expression (10) and, without it, expression of numerous genes in the developing spore is prevented. A major function of σ^F is activation of expression of the prespore specific sigma factor σ^G, which is responsible for gene expression in the developing spore (11). Moreover, the processing of pro-σ^E into σ^E, which is responsible for gene expression in the mother cell, is also blocked by nonsense mutations in *spoIIAC* (11). The deletion of the *spoIIAC* gene could possibly lead to total disruption of development in

the spore and blockage of mother cell development, which is dependent on σ^E.

For these reasons, and because previous studies had suggested that *spoIIAC* mutations may have little effect on extracellular enzyme synthesis in *B. subtilis* (15, 16) the inventors chose this as a target for deletion in *B. licheniformis*.

<u>SUMMARY OF THE INVENTION</u>

The present invention provides a process for the production of a translocated polypeptide comprising

i) cultivating a bacterium of the genus *Bacillus* which is incapable of sporulation due to a mutation, said bacterium comprising DNA constructs encoding polypeptides involved in the production of said metabolite under conditions conducive to the expression of said DNA constructs and production of said metabolite, and

ii) recovering said metabolite,

with the proviso that said bacterium does not belong to the species *B. subtilis*.

The invention furthermore provides host bacteria of the genus *Bacillus* which is incapable of sporulation due to a mutation, and a method for producing such hosts.

The method is based on the use of information provided from *B. subtilis* to enable the deletion of one or more genes involved in the sporulation process.

A deletion of the *spoIIAC* gene was prepared *in vitro* using the splicing by overlap extension technique. This gene was introduced into *Bacillus licheniformis* in a temperature sensitive plasmid and, following integration and excision from the chromosome, a precisely located deletion of the chromosomal gene was prepared.

The mutated bacterium was totally asporogenous and formed abortively disporic cells characterized by asymmetric septa at the

poles of the cell. Qualitative plate tests indicated that the bacterium synthesized normal levels of DN'ase, polygalacturonate lyase, protease, RN'ase and xylanase but hydrolysis zones due to β-1,3-glucanase and carboxymethyl cellulase activity were reduced in the mutant compared with the parent strain.

The synthesis of alkaline protease was the same in batch cultures of the mutant and parent during prolonged incubation for 72 h, but α-amylase yields were reduced by about 30% by the mutation.

In this study, we show that *in vivo* recombination can be used in *B. licheniformis* to prepare defined gene deletions and that a deletion of *spoIIAC* gives rise to a strain which is totally and stably defective in sporulation, and yet retains normal synthesis of serine protease but a slight reduction in amylase synthesis.

The invention has been exemplified in *B. licheniformis*, but it is envisaged that the invention can be used in other Bacilli as well, such as *B. lentus*, *B. amyloliquefaciens*, *B. thuringiensis*, *B. alcalophilus*, *B. mesentericus*, etc.

BRIEF DESCRIPTION OF THE TABLES AND DRAWING

Table I shows the excision frequency of pE194ts carrying the deleted *SpoIIAC* allele of *Bacillus licheniformis*

Figure 1 shows the splicing by overlap extension reaction used to create the deletion in *spoIIA*.

A. Details of the primers used (figures represent nucleotide positions of the 3'- ends of the primers A, B, C, D).

B. The sequence of the *spoIIAC* gene obtained by sequencing the PCR product generated using primers A and D from the deleted strain, DN286 *spoIIACD3*.

Figure 2 shows the integration and excision reactions resulting in the production of a deletion in *spoIIAC*.

Figure 3 shows a thin section of a typical cell from a culture of the *spoIIAC* deletion mutant showing the abortive disporic phenotype (bar = 3 mm).

Figure 4 shows the growth, sporulation and extracellular enzyme synthesis in *B. licheniformis* DN286 and DN286 *spoIIACD3*.
A and B; growth (■) and spores (□) of strain DN286 and growth of DN286 *spoIIACD3* (●) in minimal medium and brain heart infusion respectively.
C and D, serine protease synthesis by strain DN286 (■) and strain DN286 *spoIIACD3* (●) in minimal medium and brain heart infusion respectively.
E, amylase synthesis in strain DN286 (□) and in DN286 *spoIIACD3* (O), in minimal medium (□, O) and in brain heart infusion (■, ●).

DETAILED DESCRIPTION OF THE INVENTION

As indicated above the invention provides a process for the production of a translocated polypeptide comprising

i) cultivating a bacterium of the genus *Bacillus* which is incapable of sporulation due to a mutation, said bacterium comprising DNA constructs encoding polypeptides involved in the production of said metabolite under conditions conducive to the expression of said DNA constructs and production of said metabolite, and

ii) recovering said metabolite,

with the proviso that said bacterium does not belong to the species *B. subtilis*.

According to the invention it is preferred that the bacterium belongs to the group of species comprising *B. licheniformis*, *B. lentus*, *B. amyloliquefaciens*, *B. thuringiensis*, etc.

The process of the invention has been exemplified by use of a *Bacillus* that has been made incapable of sporulation through a deletion of the SpoIIAC gene, but any other mutation

effectively disrupting the sporulation process in an irreversible manner, such as the Spo2-mutation, and the Spo3-mutation is also comprised within the invention.

As indicated the process of the invention should preferably make use of a bacterium wherein said mutation is irreversible.

The process of the invention is contemplated to be used primarily for the production of any polypeptide, especially translocated polypeptides.

Said polypeptide may be endogenous to the bacterium or exogenous, meaning that the polypeptide originally was produced by itself or some other organism, respectively.

However, It is also envisaged that the sporulation deficient strains of the invention can be used for the production of secondary metabolites.

The products from the process of the invention are preferably enzymes, especially industrial enzymes, such as proteases, lipases, cellulases, oxidoreductases, amylases, etc.

Other products producible by the process of the invention are medicinal enzymes.

The term "translocated polypeptide" is intended to indicate that the polypeptide expressed carries a signal sequence which enables it to be translocated across the cell membrane. In particular, the translocated polypeptide may be a secreted polypeptide or a polypeptide involved in the secretory machinery of the *Bacillus* cell in question.

The present invention also provides a method of producing a bacterium belonging to the genus *Bacillus* which is incapable of sporulation due to a mutation whereby one or more genes involved in the sporulation process is deleted in part or completely.

Gene integration and excision is a common procedure for producing specific mutations in *B. subtilis* and other gram positive bacteria (10, 24, 25). Here we show that it is also an efficient procedure for introducing defined mutations into the chromosome of other *Bacillus*, such as *B. licheniformis*.

Given the close relationship between *B. subtilis* and *B. licheniformis* (26), it was of interest to examine the possibility of using heterologous alleles for the introduction of genes into the chromosome In this way it is according to the invention possible to use the large number of sequenced genes from *B. subtilis* for genome manipulation in other *Bacillus*, such as *B. licheniformis*. Homology was sufficient for integration of the *spoIIAC* allele of *B. licheniformis* into the *B. subtilis* chromosome, albeit at lower frequency than the homologous reaction, and integration presumably occurred at the correct site since these integrants were asporogenous.

However, excision in the *B. subtilis* host invariably occurred by a second-site recombination event at the integration cross-over point and no deletions were recovered. Presumably high homology in this region introduced a recombinational hot-spot and consequently all progeny (at least 2000 examined from several integrants) retained an intact *spoIIAC* gene.

It seems likely that the reverse situation would pertain in *B. licheniformis;* and *B. subtilis* genes forced into the *B. licheniformis* chromosome would excise perfectly.

Assuming that *B. subtilis* and *B. licheniformis* follow a similar developmental pathway, the effect of the deletion in *spoIIAC* in *B. licheniformis* can be discussed in the light of the *B. subtilis* model. Indeed, the differentiation of *B. licheniformis* DN286 *spoIIACD3* into an abortively disporic cell, exactly like that seen in *spoIIAC* mutants of *B. subtilis* (4), entirely supports this assumption.

Extracellular enzymes are generally synthesized in the later stages of exponential growth and during the early stationary phase prior to stage II of sporulation (reviewed in 27). It is therefore predictable that mutations of *spoIIAC* should have little or no effect on the expression of these genes and, indeed, with the inexplicable exception of the β-glucanases, plate tests revealed no gross changes in extracellular enzyme yield in the mutant compared with the parent strain.

Genes expressed before septation is complete will be expressed in the SpoIIA cell. This includes all stage 0 genes and some stage II genes as well as regulatory genes such as *abrB*, *hpr*, *sen* and *sin* (12). Most or all the controls affecting *aprE* expression in *B. subtilis*, of which there are at least nine independent genes or systems (12), are early stationary phase phenomena and probably function effectively in the *spoIIAC* mutant. It follows that the pattern of serine protease synthesis and enzyme yield in the mutant would match those of the parent, as seen in Fig. 4. Indeed, similar results for serine protease synthesis in *B. subtilis* have been reported by Arbidge *et al*. (13) using a non-defined asporogenous mutant.

The expression of *amyE is* subject to far fewer regulatory genes in *B. subtilis* than *aprE*. Catabolite repression and the DegSU systems are the major control systems for this gene (12), although *sen* (28) and *pai* (29) are also involved. It is not readily apparent why the synthesis of amylase was affected by the *spoIIAC* mutation.

It is possible that the early sporulation sigma factor, σ^H accumulates in the *spoIIAC* mutant because the blockage prevents σ^F and σ^E synthesis; the former due to the deletion and the latter because active σ^E production from the precursor pro-σ^E is dependent on σ^F (11).

Indeed there is evidence for enhanced accumulation of σ^H in a *spoIIA* mutant (Errington, J; personal communication). Thus the

decline in α-amylase yield may be due to the partial replacement of E-σ^A by E-σ^H.

One of the major aims of the invention is to provide a bacterium suitable for industrial enzyme production purposes. The mutant described here is totally asporogenous under laboratory conditions and the molecular biology of sporulation predicts that it should be unable to produce endospores. The likelihood of reversion of a deletion spanning almost 400 bp is minimal and there is no evidence that suppression may be a problem. Finally, the bacterium is equivalent to its parent in *e.g.* alkaline protease synthesis.

MATERIALS AND METHODS

Strains and growth conditions.
DN286 is a *Bacillus licheniformis* wild type strain

B. licheniformis NCIMB 6346 is available from NCIMB.

Bacillus subtilis 168 was obtained from D. A. Smith (University of Birmingham, UK).

Escherichia coli JM83 was used for all plasmid constructions.

Luria broth and Luria broth agar (16) were used routinely with appropriate antibiotic selections; ampicillin (100 mg/ml) and erythromycin (1 mg/ml). Spizizen's minimal salts medium (16), Schaefer's sporulation medium (16) and brain heart infusion (BHI) broth (Oxoid) were also used. Incubation was at 37°C unless otherwise stated.

Plasmids.
Plasmid pUC19 was used for cloning in *E. coli* JM83.

The complete *spoIIA* operon from *B. licheniformis* cloned in pUC13 (17) was provided by M. Yudkin (University of Oxford, UK), and

pE194ts, a mutant of pE194 which is temperature sensitive for replication was a gift from P. Youngman (University of Georgia, Athens, Ga, USA).

DNA manipulations.

Most methods followed Sambrook et al. (18) or have been described previously (21). DNA was excised from agarose gels and purified prior to ligation using the Gene-Clean II kit (Bio 101 Inc.). DNA sequencing of PCR products was performed using the automated DNA sequencing service provided by the Department of Molecular Medicine, Kings College, University of London, UK, using an ABI 373A DNA sequencer. The sequencing primer, SEQ ID NO. 1

5'-CGATCATGGAAAATTTCATGGATG-3'

was complementary to a region approximately 100 bases upstream of the proposed recombination junction, corresponding to bases 943 to 966 of the published sequence (17).

Enzyme assays.

Qualitative estimates of enzyme secretion were based on plate detection methods described previously (22).

Alkaline protease was assayed in culture supernatants using azocasein as substrate. Potassium phosphate buffer (50 mM, pH 9.0,1 ml) and culture supernatant (1.0 ml) were equilibrated at 37°C for 3 min and the reaction started by the addition of 0.5 ml 0.8% azocasein solution in phosphate buffer. At appropriate time periods, 0.5 ml reaction mixture was precipitated with 0.5 ml of ice-cold 20% TCA, the mixture centrifuged and the absorbance of the supernatant measured at 405 nm. Activity is expressed as change in absorbance per min, per ml supernatant.

Amylase was assayed in culture supernatants using Phadebas amylase substrate (Pharmacia) at 37°C and converted to release of reducing sugars (maltose) from soluble starch using a standard curve constructed with dilutions of amylase assayed by the Phadebas and Nelson-Somogyi reducing sugar assay. Units are mM maltose equivalents released/min/ml supernatant. All batch cultures were conducted at least twice; figures presented are representative of reproducible patterns of results.

Electron microscopy.
Cells grown in minimal medium containing 0.5% maltose as carbon source were collected by centrifugation and washed in 10 mM Tris buffer, pH 8 containing 50 mM NaCl. Cells were stained with aqueous uranyl acetate (4% for 2 h) and counterstained with lead citrate according to Robards and Wilson (23). Thin sections were viewed under a Jeol 100S transmission microscope.

EXAMPLES

EXAMPLE 1

Preparation of a deletion of *spoIIAC*.
The splicing by overlap extension (SOE) reaction was used (19). The promoter proximal region of the *spoIIAC* gene was amplified in a reaction with primers A (SEQ ID NO. 2);

5'-GCGGC<u>GAATTC</u>AGCTTGACCCGACGATGGATGAACTG-3'

and B (SEQ ID NO. 3);

5'-CACGACCTCTTCTGAACTGAAGTTCTTTCACTTCATGGTCTTTAAGCTG-3'

and the promoter distal region of the *spoIIAC* gene was amplified in a separate reaction with primers C (SEQ ID NO. 4);

5'-GACCATGAAGTGAAAGAACTTCAGTTCAGAAGAGGTCGTGATGGCC-3'

and D (SEQ ID NO. 5);

5'-GCGGCGGATCCTGCCTGCAACATGAGCAGCCTCAGC-3' (Fig. 1).

The underlined sequences in primers A and D represent *Eco*RI and *Bam*HI sites respectively. The reaction mixtures comprised 200 ng of template DNA (cloned *spoIIA* operon of *B. licheniformis*), 100 pmol of each primer, a final concentration of 125 mM for each dNTP, 6 ml of $MgCl_2$ (25 mM), 10 ml of (x10) Taq Buffer, and sterile Millipore water to 100 ml.

The amplification programme consisted of an initial cycle of denaturation at 95°C for 10 min after which 1 unit of Taq polymerase (Promega) was added and the reaction mixture was covered with 100 ml of light mineral oil. This was followed by 35 cycles of denaturation at 95°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. An additional cycle of 95°C/2 min, 55°C/2 min and an extension step of 72°C/6 min completed the program.

The 464 and 382 bp PCR products were concentrated by ethanol precipitation, purified using the Gene-Clean kit and used as a template (each at 300 ng) with primers A and D in the SOE reaction (same conditions as above) to generate the 806 base-pair *spoIIAC* deletion comprising fragment AD (Fig. 1).

EXAMPLE 2.

Introduction of deletion into *Bacillus licheniformis* and *B. subtilis*.

Fragment AD was cloned into pUC19 through the *Bam*HI and *Eco*RI restriction sites contained in primers A and D, respectively, transformed into *E. coli* JM83 and colonies containing plasmid with inserts screened on LB ampicillin plates containing 40 mg/ml X-gal.

Recombinant plasmid from a clone was then ligated to pE194ts through the unique *Pst*I sites in each replicon after treating the former with calf intestinal alkaline phosphatase. Ligation

mixes were transformed into *E. coli* JM83 and the recombinants detected by agarose gel electrophoresis of mini-preparations. Plasmid prepared from *E. coli* was then used to transform protoplasts of *B. licheniformis* DN286 (10) and competent cells of *B. subtilis* 168 (20, 7).

B. licheniformis and *B. subtilis* containing the pE194-based deleted *spoIIAC* allele were grown overnight in 50 ml LB broth containing erythromycin at 28°C. Samples were diluted and mixed with 3 ml of soft agar containing erythromycin and overlaid onto LB agar Em plates and incubated at 28°C (permissive for replication) and 40°C (non-permissive). The frequency of integration was the ratio of the number of colonies growing at the permissive to those growing at the non-permissive temperature. Integrants isolated at 40°C were subsequently grown routinely at 37°C.

Excision of integrated plasmid was achieved by growing cells at 30°C in LB broth without antibiotic selection. Diluted samples were plated on LB agar and incubated overnight at 37°C and colonies replica plated onto LB agar with and without erythromycin. The frequency of excision was determined as the ratio of total number of colonies to the number of Em^r colonies.

EXAMPLE 3

Preparation of a deletion in *spoIIAC*.

A deletion in the *spoIIAC* gene of *B. licheniformis* was prepared using the splicing by overlap extension (SOE) technique. Two independent PCR amplifications were made from the cloned *spoIIA* operon using the primers described in Materials and Methods. One product (AB) comprised the distal end of the downstream gene *(spoIIAB)* and the proximal region of the *spoIIAC* gene. The second product (CD) covered the distal region *of spoIIAC* and part of the upstream non-coding region. The extensive regions of homology (overlap) in primers B and C were used to initiate a third PCR amplification which comprised products AB and CD and incorporated primers A and D. This resulted in a fragment

containing the ends of the *spoIIAC* gene encompassing a deletion of 372 bp (fragment AD, see Fig. 1).

Fragment AD was cloned into pUC19 and ligated to pE194ts through the unique *Pst*I sites in each replicon to produce the shuttle plasmid pHWM2 (Fig. 2) which was transformed into *E. coli*. Plasmid prepared from *E. coli* was then used to transform protoplasts of *B. licheniformis* DN286 and competent cells of *B. subtilis* 168. Clones from each transformation were verified to contain pHWM2 by restriction enzyme analysis and used for integration and excision studies.

Example 4

Integration and excision of the deleted *spoIIAC* allele.

B. licheniformis and *B. subtilis* cells containing pHWM2 were grown overnight at 28°C and plated onto selective (Em-containing) and non-selective media and incubated at 45°C. The integration frequency, which was estimated as the proportion of cells which could grow on erythromycin plates at 45°C, for *B. licheniformis* was about 10^{-4} and for *B. subtilis* (containing the *B. licheniformis* allele) 100-fold lower (Table 1). When several integrants were tested for excision of the plasmid following activation of rolling circle replication in the integrated plasmid by culturing at 28°C, the frequency was between 10^{-2} and 10^{-3} for *B. licheniformis*.

TABLE I

Excision frequency of pE194ts carrying the deleted *Spo11AC* allele of *Bacillus licheniformis*

Organism	Inte-grant	Out-growth Period (h)	No. of Colonies Tested	Excision Frequency	Spo$^-$ Co-lonies
B. licheni-formis	1	24	150	9×10^{-2}	0
	2	24	100	2×10^{-2}	0
	3	24	150	6×10^{-2}	44
	4	24	150	7×10^{-2}	20
	5	24	150	5×10^{-2}	25
B. subtilis	1	24	560	1×10^{-1}	0
		48	114	2×10^{-1}	0
	2	24	358	1×10^{-1}	0
		48	152	2×10^{-1}	0
	3	24	442	2×10^{-1}	0
		48	184	3×10^{-1}	0

In two clones (integrants 1 and 2) excision was invariably through the integration site and wild type, Spo$^+$ progeny were recovered. However, for the other three clones excision was also through a second site recombinational event (see Fig. 2) giving rise to deletions of the *spoIIAC* gene in the Em^S derivatives. Interestingly, the insertion was very unstable in *B. subtilis* but invariably excision occurred at the insertion point and no Spo$^-$ progeny were recovered even after a prolonged outgrowth period of 48 h. (Table I).

Example 5

Characterization of *B. licheniformis DN286 spoIIACD3*.

A deletion strain from *B. licheniformis* integrant 3 (Table 1) was chosen for further study. Hybridization of a labelled *spoIIA* operon from *B. licheniformis* to Southern blotted chromosomal DNA from the wild type and deleted strains which had been cut with EcoRI, revealed a smaller hybridizing

fragment of 3.8 kb in the mutant compared with 4.2 kb in the parent (data not shown). PCR amplification from chromosomal DNA of the mutant and parent strains using primers A and D showed that the mutant possessed a deletion of about 370 bp. When the PCR product from the mutant was sequenced, a deletion located precisely at the junction point between primers B and C was revealed (Fig. 1B). Morphological confirmation of a *SpoIIA* mutation was provided by electron microscopy. The two asymmetric septa in an "abortively disporic" cell typical of a *B. subtilis spoIIAC* mutant were clearly visible (Fig. 3).

The deletion gave rise to completely asporogenous cultures in which spores were undetectable. After growth in BHI, minimal medium (Fig. 4) and Schaeffer's sporulation medium (data not shown) for 72 h the sporulation frequency of the parent was about 2% in each case.

The naturally low sporulation rate of DN286 was confirmed by comparing with *B. licheniformis* NCIMB 6346 which showed 90% to 100% sporulation under the same three conditions.

Spores were never detected in the *spoIIAD3* derivative in any of the media. Despite the absence of spores, there was no evidence for significant loss of viability or cell lysis in the mutant strain when grown in BHI and the cell population remained steady at about 4×10^9 cfu/ml throughout the incubation period. In minimal medium, however, there was some loss of viability when the mutant was incubated beyond 36 h, and the final cell population approached 3×10^8 cfu/ml after peaking 10-fold higher. Loss of viability was less pronounced in the parent strain growing in minimal medium.

Example 6

Extracellular enzyme synthesis in *B. licheniformis DN286 spoII-ACD3*.

Initial plate tests suggested that the mutant strain showed some minor differences from the parent in the amounts of extracellular enzyme synthesized. In particular, hydrolysis

zones from β-1,3-glucanase and carboxymethyl cellulase were reduced in the mutant compared with the parent strain but synthesis of a-amylase, DN'ase, polygalacturonate lyase, protease, RN'ase and xylanase was largely unaffected.

The mutant and its parent were grown in rich (BHI) and minimal media for 72 h. Sporulation and the synthesis of serine protease were monitored (Fig. 4). Protease production was similar in both parent and mutant. In minimal medium, enzyme yield peaked after incubation for about 40 h in both strains and then remained steady. In BHI, enzyme yield was slightly repressed and peaked after incubation for about 48 h in both strains. The subsequent decline in enzyme yield was less pronounced in BHI than in the salts medium. In both media, the yield of serine protease was unaffected by the *spoIIAC* deletion.

α-Amylase synthesis consistently initiated later during batch culture of the mutant compared to the parent and consequently these fermentations were continued for a longer time period than those for protease (84 h). In both media, the yield of enzyme from the mutant was about 70% of that obtained from the parent strain. However, if the enzyme yields were expressed as specific activities per unit of biomass, the mutant performed almost as well as the parent, particularly later in the growth cycle when biomass declined in the minimal medium.

The present invention has been illustrated through the examples which are only showing specific embodiments thereof. This should not be construed as limiting the invention thereto, since many other embodiments will be obvious to the skilled person upon reading it.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: DENMARK
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4442 2668
(H) TELEFAX: +45 4442 6080

(ii) TITLE OF INVENTION: Process for producing useful proteins

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Probe"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..24

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Probe"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..37

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Probe"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..49

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(B) LOCATION:1..46

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

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(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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PATENT CLAIMS

1. A process for the production of a metabolite comprising

i) cultivating a bacterium of the genus *Bacillus* which is incapable of sporulation due to a mutation, said bacterium comprising DNA constructs encoding polypeptides involved in the production of said metabolite under conditions conducive to the expression of said DNA constructs and production of said metabolite, and

ii) recovering said metabolite,

with the proviso that said bacterium does not belong to the species *B. subtilis*.

2. The process of claim 1, wherein said bacterium belongs to the group of species comprising *B. licheniformis*, *B. lentus*, *B. amyloliquefaciens*, *B. thuringiensis*.

3. The process of claim 1 or 2, wherein said mutation is selected from the group of mutations comprising the Spo2-mutation, the Spo3-mutation, the SpoIIAC-mutation.

4. The process of any of the claims 1 to 3, wherein said mutation is irreversible.

5. The process of any of the claims 1 to 4, wherein said metabolite is an endogenous or exogenous polypeptide.

6. The process of claim 5, wherein said polypeptide is translocated.

7. The process of claim 5 or 6, wherein said polypeptide is an enzyme.

8. The process of any of claims 5 to 7, wherein said enzyme is an industrial enzyme.

9. The process of any of claims 5 to 7, wherein said enzyme is a medicinal enzyme.

10. A method of producing a bacterium belonging to the genus *Bacillus* which is incapable of sporulation due to a mutation whereby one or more genes involved in the sporulation process is deleted in part or completely.

11. The method of claim 10, wherein said bacterium belongs to the group of species comprising *B. licheniformis*, *B. lentus*, *B. amyloliquefaciens*, *B. thuringiensis*.

12. The method of claim 10 or 11, wherein said mutation is selected from the group of mutations comprising the Spo2-mutation, the Spo3-mutation, the SpoIIAC-mutation.

13. The method of any of the claims 10 to 12, wherein said mutation is irreversible.

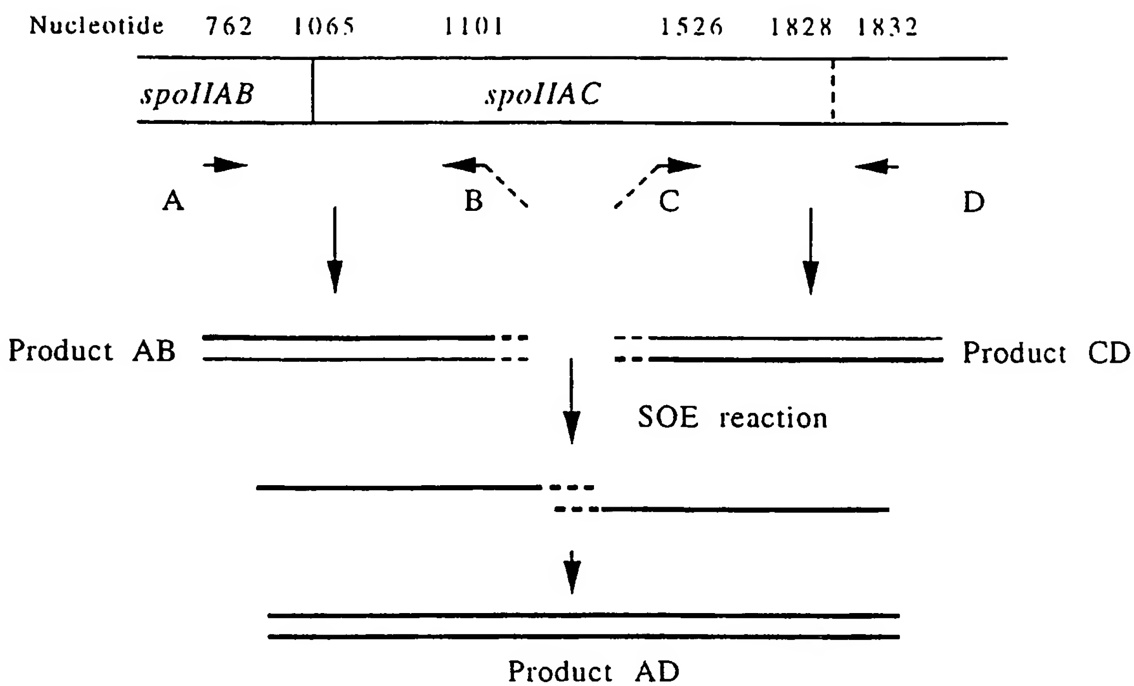
14. A bacterium belonging to the genus *Bacillus*, except for *B. subtilis*, which is incapable of sporulation due to a mutation.

15. The bacterium of claim 14, wherein said bacterium belongs to the group of species comprising *B. licheniformis*, *B. lentus*, *B. amyloliquefaciens*, *B. thuringiensis*.

16. The bacterium of claim 14 or 15, wherein said mutation is selected from the group of mutations comprising the Spo2-mutation, the Spo3-mutation, the SpoIIAC-mutation.

17. The bacterium of any of the claims 14 to 16, wherein said mutation is irreversible.

A. SOE Reaction



B. Sequence

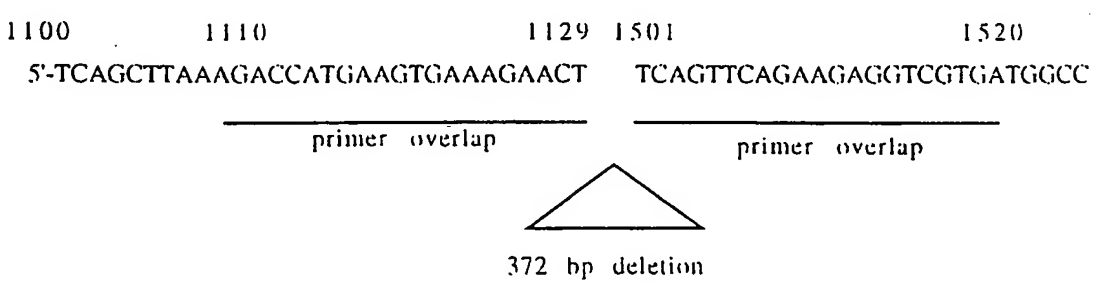
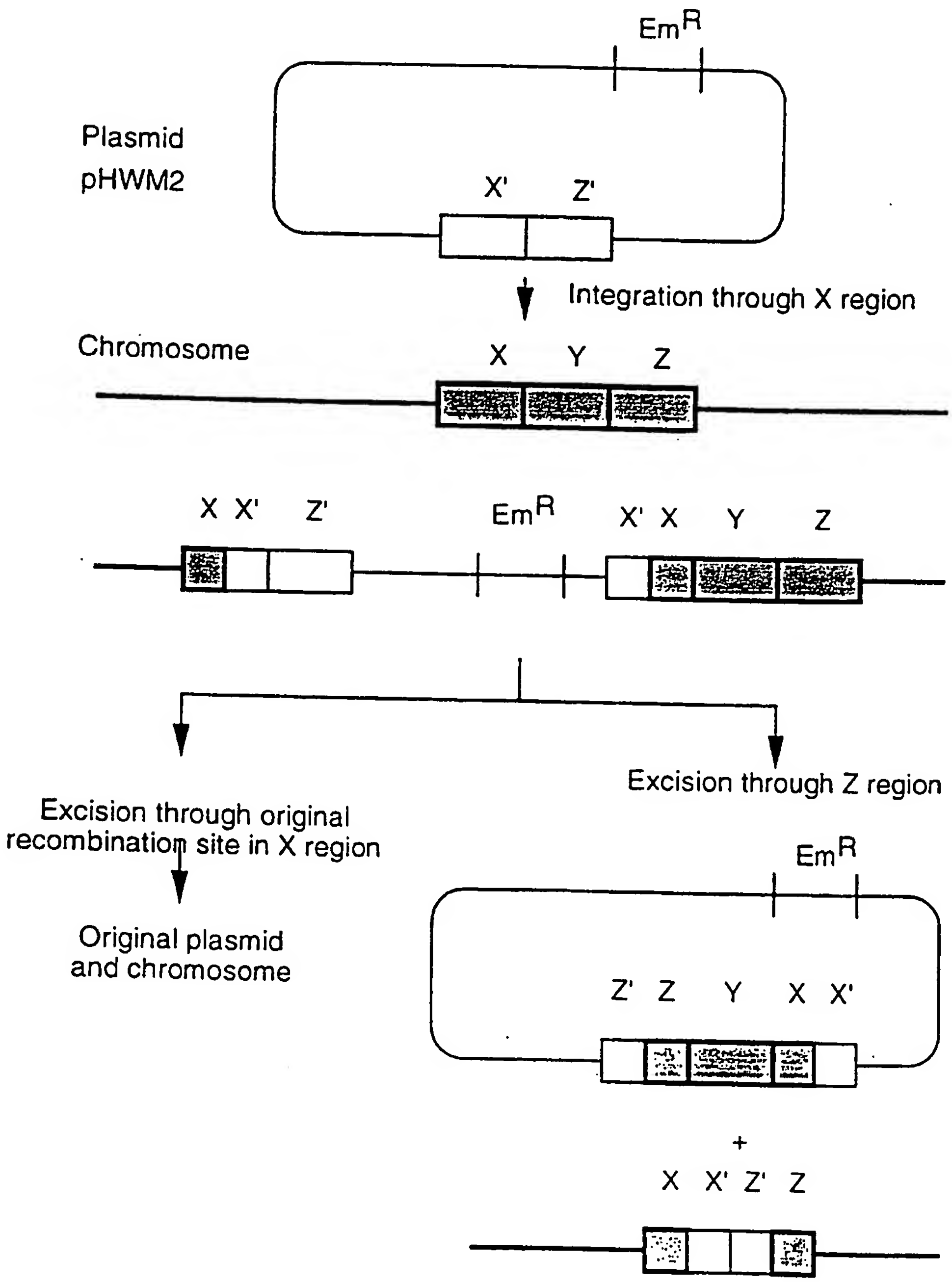


FIG. 1



EmR
Plasmid
pHWM2
X'
Z'
Integration through X region
Chromosome
X
Y
Z
X X' Z'
EmR
X' X Y Z
Excision through original recombination site in X region
Excision through Z region
Original plasmid and chromosome
EmR
Z' Z Y X X'
+
X X' Z' Z

FIG. 2

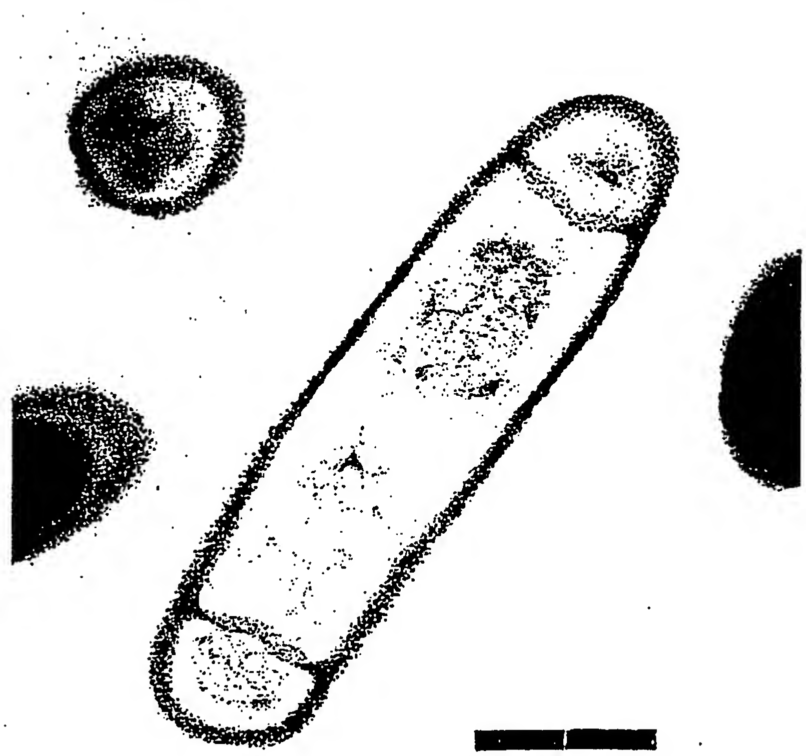


FIG. 3

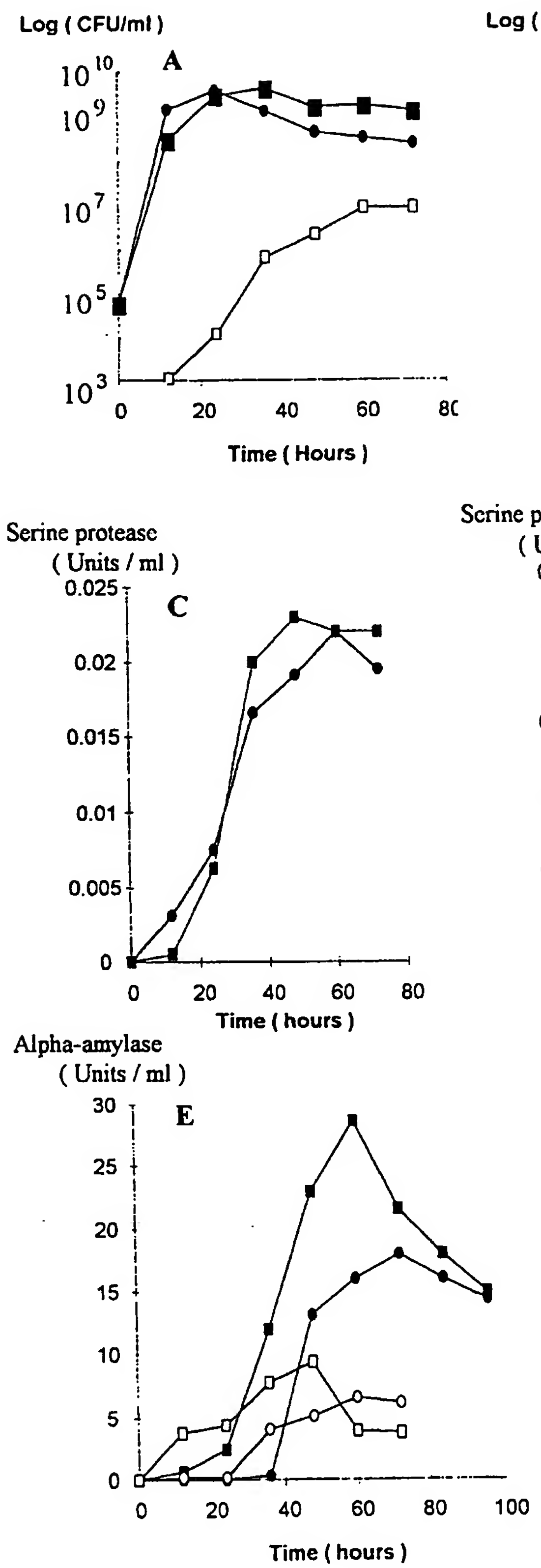

Log (CFU/ml)
A
10^{10}
10^{9}
10^{7}
10^{5}
10^{3}
0 20 40 60 80
Time (Hours)
Log (CFU/ml)
B
0 20 40 60 80
Time (hours)
Serine protease
(Units / ml)
C
0.025
0.02
0.015
0.01
0.005
0
Time (hours)
D
Alpha-amylase
(Units / ml)
E
30
25
20
15
10
5
0
0 20 40 60 80 100
Time (hours)

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00304

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 1/21, C12N 9/00, C12P 21/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, PAJ, WPI, MEDLINE, BIOSIS, DBA, CA, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, File 34, SciSearch, Dialog accession no. 14311191, Fleming AB et al: "Extracellular Enzyme-Synthesis in a Sporulation -Deficient Strain of Bacillus-Licheniformis"; Applied and Environmental Microbiology, 1995, V61, N11 (NOV), p 3775-3780 --	1-17
X	EP 0369817 A2 (BIOTECHNICA INTERNATIONAL, INC.), 23 May 1990 (23.05.90), page 1, line 50 - line 54; page 2, line 10 - line 13, claims --	1-17
X	WO 8904866 A1 (CELLTECH LIMITED), 1 June 1989 (01.06.89), claims -- --------	1-17

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 1996	17.10.96
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/09/96

International application No.
PCT/DK 96/00304

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0369817	23/05/90	AT-T- 137265	15/05/96
		AU-A- 4476689	24/05/90
		CA-A- 2003078	18/05/90
		DE-D- 68926333	00/00/00
		JP-A- 3067582	22/03/91
WO-A1- 8904866	01/06/89	AU-A- 2727388	14/06/89
		EP-A- 0351427	24/01/90
		JP-T- 2502247	26/07/90